Reference: *Biol. Bull.*, **138**: 326–333. (June, 1970).

INACTIVATION OF α-ECDYSONE AND CYASTERONE BY LARVAE OF THE FLESHFLY, *SARCOPHAGA PEREGRINA*, AND PUPAE OF THE SILKWORM, *SAMIA CYNTHIA*[1]

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Certain weeds, ferns, and evergreen trees contain complicated sterols closely resembling the insect hormones, α- and β-ecdysones (Fig. 1). When injected into insects, many of these so called "phytoecdysones" have proved to be more active than the insects' own hormones.

A case-in-point is cyasterone, a phytoecdysone which Takemoto, Hikino, Nomoto and Hikino (1967) isolated from the roots of *Cyathula capitata* (Amaranthaceae). As shown in Figure 1, cyasterone is characterized by the presence of a lactone ring at the end of its side-chain. When assayed on mature larvae of the fleshfly, *Sarcophaga peregrina*, cyasterone was twice as active as α-ecdysone (Ohtaki, Milkman and Williams, 1967). In assays on the bee, *Nomia melanderi*, it was 10 times as active (Hsiao and Hsiao, 1969). In assays on pupae of the silkworm, *Samia cynthia*, it was 20 times as active as α-ecdysone and 40 times as active as β-ecdysone (Williams, 1968).

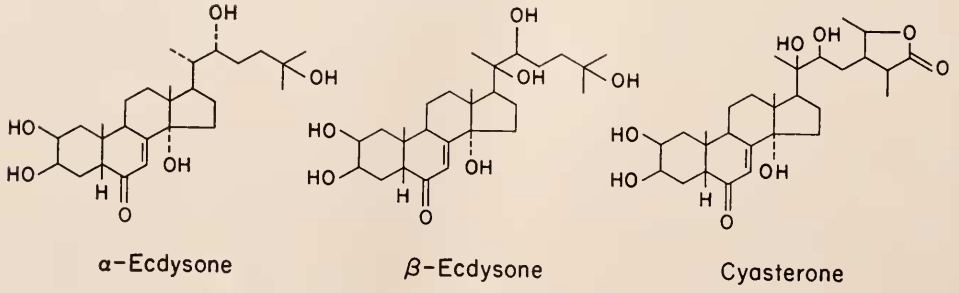

FIGURE 1. Structural formulae of the authentic hormones, α- and β-ecdysones, and the phytoecdysone, cyasterone. The stereochemistry of the side-chain is known only for α-ecdysone.

The greater potency of the hormonal analogue is a phenomenon well known to students of mammalian endocrinology. Especially in the case of the sterol hormones of the gonads and adrenal cortex, amplification of activity has frequently been achieved by chemical modifications of the authentic hormones. The enhanced activity can often be accounted for in terms of the greater resistance of the analogues to enzymes attacking the native hormone (Steelman and Hirschmann, 1967).

[1] This study was supported, in part, by NSF Grant No. GB-7963.

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In the present investigation we have sought to determine whether this simple explanation suffices in the case of cyasterone. To this end we have studied the decay of hormonal activity in two species of insects injected with cyasterone or α-ecdysone.

Materials and Methods

1. Experimental animals

Larvae of *Sarcophaga peregrina* were reared as previously described (Ohtaki, Milkman and Williams, 1968) and utilized as mature larvae or as "standard test abdomens." Additional experiments were performed on diapausing larvae of the silkworm, *Samia cynthia* (weight 2.5 g), which were purchased from dealers and stored at 25° C. One series of experiments made use of pupae of the silkworm, *Hyalophora cecropia* (weight 5 g).

2. Ecdysones

Synthetic α-ecdysone was obtained from Dr. John Siddall, of the Zoecon Corporation. A weighed sample was dissolved in isopropanol and diluted with 9 parts water. The stock solution was further diluted with 10% isopropanol. Parallel experiments were carried out on cyasterone; the latter was obtained from Professor T. Takemoto, of Tohoku University.

3. Injections, extractions, and biological assays

Since all these procedures have already been described in detail (Ohtaki *et al.*, 1968), it suffices to outline the overall plan of the experiments reported here.

Our objective was to measure the "half-inactivation time" of α-ecdysone and cyasterone in fly larvae and silkworm pupae. To this end, each of a series of animals was injected with a certain dose of α-ecdysone or cyasterone. Thereafter at successive intervals, one or more individuals was sacrificed and extracted with a solvent mixture suitable for the recovery of ecdysones. The extract was purified to eliminate toxic water-soluble materials and then subjected to biological assay on standard test abdomens of *Sarcophaga peregrina*. The assay had been calibrated by the injection of known amounts of α-ecdysone or cyasterone. It was therefore possible to equate the endocrine activity of each extract with the activity of a corresponding amount of α-ecdysone or cyasterone. By plotting these values as a function of time, the half-inactivation time was estimated.

The apparent simplicity of these experimental maneuvers is deceptive. For example, we know the identity and amount of hormone injected at zero time; we also know its biological activity as defined by the puparium assay. But during the succeeding hours we know neither the identities nor the amounts of the active materials which we extract, purify, and assay. However, by means of the puparium assay, their combined activity can be determined and equated to that of a certain concentration of α-ecdysone or cyasterone. In short, what we measure is, not the decay of the hormone injected at zero time, but the decay of endocrine activity generated by that injection. As long as the purified extract harvests all materials with ecdysone activity, our measurements are independent of the precise

identities of these materials. By suitable tests it was possible to show that this requirement was apparently satisfied in the present study.

Results

1. *Experiments on* Sarcophaga peregrina

The top section of Table I records the per cent of initial activity recovered 0, 1, 3, and 8 hours after the injection of 1 μg α-ecdysone into 0-hour larvae. The hormone in each case was extracted, purified, redissolved in distilled water, and assayed on 10 standard test abdomens. The average puparium index was calculated and converted to micrograms as described by Ohtaki *et al.* (1968). In the right hand column of Table I the recoveries have been corrected for 10% loss in

Table I

Decay of ecdysone activity in mature larvae of Sarcophaga peregrina *injected with α-ecdysone or cyasterone*

Substance injected*	Number of larvae injected	Time between injection and extraction (hrs)	% of activity recovered	Corrected % recovery
α-ecdysone	1	0	88	97
	1	0	102	113 } 99
	1	0	78	87
	1	1	31	34
	1	1	53	59 } 51
	1	1	53	59
	2	3	27	30
	2	3	20	22 } 23
	2	3	16	18
	4	8	1	1
	4	8	3	3 } 2
	4	8	1	1
Cyasterone	1	0	88	97
	1	0	92	102 } 97
	1	0	83	92
	1	1	72	80
	1	1	84	93 } 78
	1	1	56	62
	2	3	46	51
	2	3	52	58 } 55
	2	3	28	31
	2	3	72	80
	3	8	9	10
	3	8	8	9 } 13
	3	8	19	21

* Each larva received 1 μg hormone in 5 μl of 10% isopropanol.

the extraction and purification procedures. The lower section of Table I summarizes similar experiments performed on *Sarcophaga* larvae injected with 1 μg cyasterone.

In Figure 2 the averaged and corrected recoveries in both experiments have been plotted as a function of the time that elapsed between injection and extraction. Despite the considerable scatter among the individual measurements, it is clear that both hormones are rapidly metabolized by *Sarcophaga* larvae, the half-inactivation time being approximately 1 hour for α-ecdysone and 3–4 hours for cyasterone.

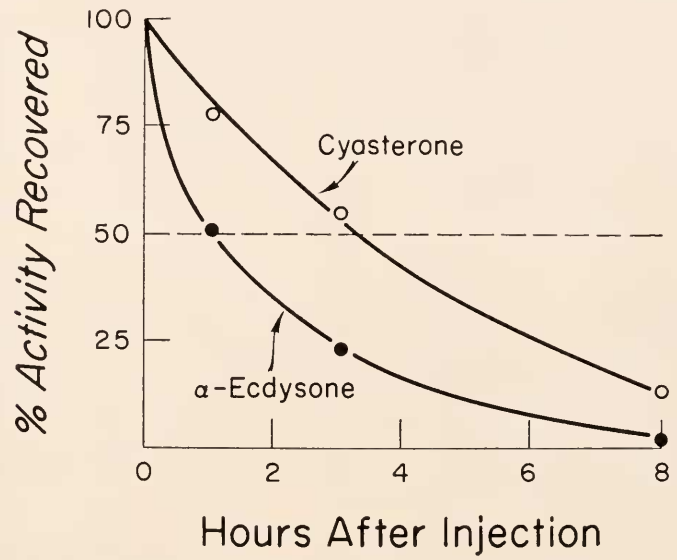

FIGURE 2. The decay of endocrine activity after the injection of 1 μ of cyasterone or α-ecdysone into mature larvae of *Sarcophaga peregrina*.

2. *Experiments on* Samia cynthia

The experiment was repeated on diapausing pupae of the Cynthia silkworm. At zero time each pupa was injected with 5 μg α-ecdysone or cyasterone in 25 μl of 10% isopropanol. The results, corrected for 20% loss of hormone in the extraction of these large insects, are summarized in Table II and Figure 3. Clearly, both materials are much more stable in silkworm pupae than in *Sarcophaga* larvae. Though the results are here again complicated by considerable scatter, the half-inactivation time for α-ecdysone appears to be about 6 hours. In the case of cyasterone, about 60% of initial activity was still present at the 24th hour; by extrapolation we estimate a half-inactivation time of approximately 32 hours.

3. *Experiments on* Hyalophora cecropia

Inactivation of α-ecdysone was also studied on freshly pupated Cecropia silkworms, each of which was injected with 5 μg. As indicated in Figure 3, α-ecdysone was inactivated at the same rate by both species of Lepidoptera.

TABLE II

Decay of ecdysone activity in diapausing pupae of the Cynthia *silkworm injected with α-ecdysone or cyasterone*

Substance injected	Time between injection and extraction* (hrs)	% of activity recovered	Corrected % recovery
α-ecdysone	0	96	120
	0	74	93 } 102
	0	74	93
	1	64	80
	1	80	100 } 82
	1	52	65
	8	40	50
	8	41	51 } 40
	8	16	20
	24	9	11
	24	16	20 } 18
	24	19	24
Cyasterone	0	68	85
	0	88	110 } 100
	0	84	105
	1	88	110
	1	76	95 } 95
	1	64	80
	8	60	75
	8	88	110 } 85
	8	56	70
	24	40	50
	24	72	90 } 62
	24	36	45

* Each extract was prepared from a single pupa that had been injected with 5 μg of hormone dissolved in 25 μl of 10% isopropanol.

DISCUSSION

1. *Metabolism of α-ecdysone*

In the present and previous studies (Ohtaki *et al.*, 1968) mature larvae of *Sarcophaga peregrina* showed an extremely rapid metabolism of α-ecdysone. Thus, after the injection of 1 μg α-ecdysone, 50% of activity was lost during the first hour and 98% during the first 8 hours. The lower dose of 0.5 μg was metabolized even faster, about 80% of initial activity being lost during the first hour. Inactivation was blocked by low temperatures or anaerobic conditions—a finding that implicates enzymatic attack on the hormone, presumably by one or more "mixed-function oxidases" (Mason, 1965; Kamin and Masters, 1968; Sih, 1969).

These results are therefore in accord with the rapid inactivation of α-ecdysone by the blowfly, *Calliphora erythrocephala*. For example, when α-ecdysone was

injected into mature larvae, 80% of activity disappeared in 2 hours (Karlson and Bode, 1969). In similar experiments performed on ligatured abdomens, the half-inactivation time was 3 hours (Shaaya, 1969).

In additional studies of *C. erythrocephala,* Karlson and Bode (1969) extracted from larval fat-body a soluble enzyme system capable of inactivating α-ecdysone *in vitro.* The titer of this system was shown to undergo large and systematic changes during successive phases of metamorphosis.

In experiments performed on *Calliphora vicina,* King and Siddall (1969) observed a much slower inactivation of α-ecdysone than noted in the above mentioned studies of *C. erythrocephala* and *S. peregrina.* For example, when tritiated α-ecdysone was injected into mature larvae, no less than 25% of the tritium label was recovered 12–24 hours later in two materials identified as α- and β-ecdysones. Evidently, the dynamics of ecdysone metabolism can show substantal differences even among closely related species.

In the present investigation a relatively slow inactivation of α-ecdysone was evident in experiments performed on the two species of silkworm pupae. As illustrated in Figure 3, the half-inactivation time was about 6 hours, and 15–20% of initial activity remained after 24 hours.

According to King and Siddall (1969), the first step in the metabolism of α-ecdysone is hydroxylation at C-20 to form β-ecdysone; the experiments in question made use of tritiated α-ecdysone and were performed on *Calliphora vicina* and two species of crustaceans. Subsequently, this finding has been confirmed by Cherbas and Cherbas (1970) in detailed studies of the metabolism of tritiated α-ecdysone by diapausing pupae of the silkworm, *Antheraea polyphemus.* These investigators report, moreover, that the further metabolism of β-ecdysone is accom-

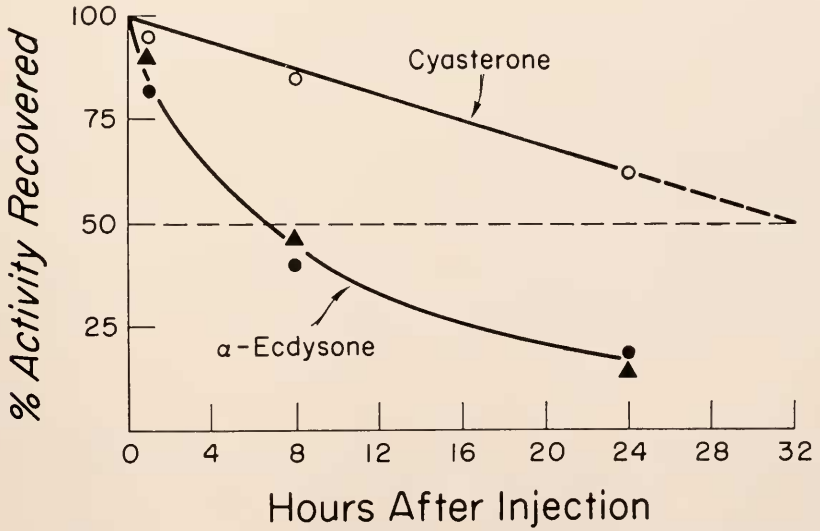

FIGURE 3. The decay of endocrine activity after the injection of 5 μg of cyasterone or α-ecdysone into diapausing pupae of the silkworms, *Samia cynthia* (○,●) or *Hyalophora cecropia* (▲).

panied by the appearance of three materials of increasing polarity but of presently unknown structure or biological activity. Substantially the same results were obtained by Moriyama, King, Nakanishi, Okauchi, Siddall and Hafferl (1970) in a study of the metabolism of tritiated α-ecdysone by mature larvae of the silkworm, *Bombyx mori*.

2. Comparison of α-ecdysone and cyasterone

Table III summarizes the parameters of these hormonal materials as ascertained in the present investigation. In the experiments on *Sarcophaga* larvae, cyasterone was twice as active as α-ecdysone and 3–4 times as stable in terms of its half-inactivation time. Therefore, cyasterone's stability can fully account for its greater activity in the fly larvae.

TABLE III

Parameters of two ecdysones in fly larvae and silkworm pupae

	Critical dose* (μg/g live weight)	Activity relative to α-ecdysone	Half-inactivation time (hrs)
Sarcophaga peregrina			
α-ecdysone	0.6	1	1
Cyasterone	0.3	2	3–4
Samia cynthia			
α-ecdysone	2.5	1	6
Cyasterone	0.1	25	32

* Dose provoking a puparium index of 50% in standard test abdomen of *Sarcophaga peregrina*; in the case of Cynthia pupae, the stated dose caused termination of diapause and development of a normal moth.

The bottom half of Table III summarizes the corresponding data for pupae of *Samia cynthia*. Cyasterone proves to be 25 times as active as α-ecdysone but only 5 times as stable in terms of half-inactivation time. So, unlike the situation in *Sarcophaga*, resistance to inactivation can only partially account for the great activity of cyasterone in the lepidopteran. The residual difference must be attributed to other presently unknown properties of the cyasterone molecule which further amplify its activity for silkworm pupae but not for the larval fly.

We are indebted to Professor T. Takemoto of Tohoku University for supplying cyasterone, and to Dr. John Siddall of the Zoecon Corporation for providing synthetic α-ecdysone. We are also grateful to Professor Lynn M. Riddiford and to Lucy and Peter Cherbas for advice and criticism of the manuscript.

SUMMARY

1. The phytoecdysone, cyasterone, is twice as active as authentic α-ecdysone when injected into mature larvae of the fleshfly, *Sarcophaga peregrina*. It is 25 times as active as α-ecdysone when injected into diapausing pupae of the silkworm, *Samia cynthia*.

2. By the biological assay of hormone extracted from injected animals, it was possible to approximate the half-inactivation times of the endocrine activities provoked by the injection of α-ecdysone or cyasterone.

3. In the fly larva the half-inactivation time was about 1 hour for α-ecdysone and about 3–4 hours for cyasterone. Therefore, cyasterone's resistance to inactivation can fully account for its 2-fold greater activity in the larval fly.

4. In diapausing silkworm pupae the half-inactivation time was about 6 hours for α-ecdysone and 32 hours for cyasterone. Consequently, cyasterone's 5-fold greater stability can only partially account for its 25-fold greater activity in the pupal silkworm.

5. The residual difference must be due to other presently unknown properties of the cyasterone molecule which further amplify its activity for silkworm pupae but not for the larval fly.

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